REMARKS/ARGUMENTS

Claims 21-23 are pending in the application. Claims 21-23 are rejected. Claims 21-23 have been amended. Entry of the amendment, reconsideration of the rejection, and allowance of claims 21-23 are requested.

The Amendment

In order to expedite prosecution of the application and advance the case toward allowance, claims 21-23 have been amended. No new matter was added by the amendment.

Claim 21-23 have been amended to remove redundant language as suggested by the Examiner.

Objections

Priority

The specific reference to priority has been amended in the specification to refer to all priority application and to indicate the correct relationship between the applications.

Oath/Declaration

The office action indicates that the oath or declaration is defective because it does not identify the *mailing address* of each inventor. The mailing address of each inventor has been provided in the attached application data sheet.

Double Patenting

Claims 21-23 are rejected under the judicially created doctrine of obviousness type double patenting as being unpatentable over claims 1-4 of U.S. Patent No. 6,479,642 B1.

Applicants have submitted as *terminal disclaimer* in compliance with 37 C.F.R. §1.321(c) to overcome the rejection since the conflicting patent is commonly owned with this application.

Rejection under 35 U.S.C. §112

Claim 22 is rejected under 35 U.S.C. §112, first paragraph, for allegedly lacking enablement. The office action indicates that the specification, while being enabling for pharmaceutical compositions comprising the specific isolated and purified cortistatin polypeptides of SEQ ID NO: 26, or structurally and functionally defined fragments thereof, does not allegedly provide enablement for any biological functional equivalent proteins with no defined structural characteristics. The Examiner asserts that the specification does not teach which particular amino acids are critical for any cortistatin polypeptide's function, nor how to distinguish such from any different polypeptide sequence that possesses none of the desired functions. In support of this argument, the Examiner cites Rudinger *et al.*, a publication from 1976 that alleges that "it is impossible to attach a unique significance to any residue in a sequence [and that] a given amino acid will not by any means have the same significance in different peptide sequences, or even in different positions of the same sequence."

The rejection is respectfully traversed.

The priority of the instant invention goes back to 1996 at which time the state of the art had already progressed to understanding the importance of conserved protein sequences across species. For example, Sekido *et al.* studied a gene for the transcriptional repressor deltaEF1 and found that the deduced amino acid sequence of deltaEF1 was highly conserved between chicken and mouse, not only in DNA-binding motifs but also in other blocks (78% overall amino acid identity) (see page 230, column 1 of attached publication of Sekido *et al.* (1996) *Gene* 173(2):227-232). Furthermore, cDNAs coding for proteins very similar to deltaEF1 had been cloned from human and hamster, and the amino acid sequences of these proteins aligned well with those of chicken and mouse deltaEF1 and DNA-binding motifs were organized identically (see page 230, column 2 of attached publication of Sekido *et al., supra).*

Contrary to the Examiner's assertion, the specification does teach which amino acids are critical for any cortistatin polypeptide's function, or how to distinguish such from any different polypeptide sequence. The specification states on page 17, lines 5-24:

> "Cortistatin has been cloned, sequenced and characterized from a variety of mammalian species, indicating that it is a neuropeptide found in all mammals,

including humans, rodents, mice, and the like mammals. The neuropeptide is not identical in amino acid residue sequence between mammalian species, but is sufficiently similar that allows generalizations regarding function, and assures that one can identify and isolate the cortistatin gene in any mammalian species. Thus, variations at both the amino acid and nucleotide sequence level are described in isolates of cortistatin, and such variations are not to be construed as limiting. For example, allelic variation within a mammalian species can tolerate a several percent difference between isolates of a type of cortistatin, which differences comprise non-deleterious variant amino acid residues. Thus, a protein of about 95% homology, and preferably at least 98% homology, to a disclosed cortistatin is considered to be an allelic variant of the disclosed cortistatin, and therefore is considered to be a cortistatin of this invention."

The Applicants have cloned, sequenced and characterized rat, mouse and human cortistatin, and shown that rat and mouse proteins share 82% identity (page 20, lines 12-14), and that rat and human nucleotide sequences share 71% identity (page 21, lines 16-17). In fact, the Applicants have provided sequences for rat, mouse and human cortistatin, examples that show the procedures used to isolate the rat, mouse and human cortistatin genes and deductions of the amino acid sequences (page 84-91), as well as characteristics of the physiology of cortistatin (page 115). The Applicants refer the Examiner to Table 1 (page 95) which lists the several rat-, mouse-, and human cortistatin proteins and peptides of the invention. As such, the determination of other mammalian cortistatin polypeptide sequences would be routine for the skilled artisan. While it might require some experimentation to arrive at other mammalian cortistatin polypeptide sequences used in a pharmaceutical composition, the quantity of experimentation required would not be undue, particularly in light of the guidance provided in the instant specification. "The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation" (*In re Certain Limited -- Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom., Massachusetts Institute of Technology v. A. B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985).

In light of the above remarks, Applicants respectfully request that the rejection of claim 22 under 35 U.S.C. §112, first paragraph, be withdrawn.

Claims 21-23 are rejected under 35 U.S.C. §112, second paragraph, for being allegedly indefinite. The Examiner suggests to amend claims 21 and 23 such that confusing and redundant language is removed in order to more accurately claim the instant invention.

Claims 21 and 23 have been amended according to the Examiner's suggestions.

In light of this amendment, the Applicants respectfully request that the rejection of claims 21 and 23 under 35 U.S.C. §112, second paragraph, be withdrawn.

Rejection under 35 U.S.C. §102

Claims 21-23 are rejected under 35 U.S.C. §102(a) as being allegedly anticipated by Fukusumi *et al.* (March 6, 1996).

The office action alleges that no human cortistatin polypeptides were described in the parent application Serial No. 08/648,322 (now U.S. Patent No. 6,074,872), and thus, priority is held to be the filing date of parent application Serial No. 08/857,389 (5/17/97) (now U.S. Patent No. 6,479,642 B1).

The office action further asserts that Fukusumi *et al.* teach isolation and purification of human cortistatin of SEQ ID NO: 26 (page 158, Figure 1) in pharmaceutical compositions (page 158, first column) that include the pharmaceutically acceptable carrier, AcONH4 buffer (pH 8) and also comprise the pharmaceutically acceptable carrier, H_2O, in dosages of between 10 pM and 1μM of cortistatin (Figure 4) as well as dosages of 0.1 to 1nmol/brain (Figure 7 and page 162). The office action further alleges that this dosage is between 50μg to abut 750mg, thereby meeting the limitations of the claims.

The rejection is respectfully traversed.

I. Priority:

Contrary to the assertion in the office action, the parent application Serial No. 08/648,322, U.S. Patent No. 6,074,872 (filed May 15, 1996) *does* teach human cortistatin. For example, the specification teaches the following in column 8, lines 5-8:

> "Cortistatin has been cloned, sequenced and characterized from a variety of mammalian species, indicating that it is a neuropeptide found in all mammals, **including humans**, rodents, mice, and the like mammals." [Emphasis added.]

The specification teaches the following in column 9, lines 37-41:

> "In view of the **conserved domains** and cleavage sites for generating mature cortistatin proteins for two mammals, rats and mice, similar cleavage patterns and resultant protein species are identifiable in other mammals **including humans**." [Emphasis added.]

Furthermore, the specification teaches how rat, mouse and human cortistatin sequences can be obtained (see column 39, lines 46-50):

> "C. Human cDNA
> The human homolog is similarly obtained from screening human brain cDNA libraries essentially as described above for rat and mouse cortistatin nucleic acids and proteins."

The specification also indicates that amino acid sequences for rat, mouse and human are the same (see Table 1 in column 41, lines 20-35), evidencing that Applicants knew in 1996 that there is a significant degree of homology among human-, rat-, and mouse cortistatin. Since Applicants have provided enough information about human cortistatin in order to isolate and clone it, the priority of pharmaceutical compositions of cortistatin should be extended to parent application 08/648,322, U.S. Patent No. 6,074,872 (filed May 15, 1996).

The Applicants further point the Examiner to the priority application Serial No. 08/648,322, U.S. Patent No. 6,074,872, wherein the Applicants have described how cortistatin-

14 peptide was infused into the brain ventricles of rats (see column 51, lines 10-20). Figure 7 showed the effects of cortistatin on the sleep wake cycle of the rats. In fact, column 51, lines 10-63 clearly describe how pharmaceutical compositions of cortistatin were tested. In light of the fact, that Applicants also taught how to isolate and clone human cortistatin, one of skill would have no difficultly testing the substantially similar human cortistatin in the same pharmaceutical compositions as taught in the priority application. Hence, the Applicants are entitled to their earliest priority date.

II. Declaration under 37 C.F.R. §1.131:

The Applicants further refer the Examiner to parent case Application Serial No. 08/857,389 (now U.S. Patent No. 6,479,642 B1, issued on November 12, 2002). In the file history of this case, the Applicants submitted a declaration under 37 C.F.R. §1.131 and were able to establish conception of cortistatin proteins and peptides (*i.e.*, rat-, mouse-, and human cortistatin) to be prior to the publication of Fukusumi *et al.* (March 6, 1997). A copy of the declaration and accompanying exhibits are attached hereto for the Examiner's convenience. The copy of Exhibit A shows relevant laboratory pages; on page 12 (*i.e.*, the last page prior to the last page) the Applicants grew cultures that contained the cortistatin clones and prepard standard minipreps (Qiagen, 500 preps of 100μl). The clones were precipitated with isopropanol and then ammonium hydroxide (NH_4OH) and Ethanol (EtOH); and resuspended in H_2O. **Notably, H_2O qualifies as a pharmaceutically acceptable carrier as the Examiner indicated by relying on Fukusumi *et al.*** In fact, the Examiner indicated the following:

> "...Fukusumi *et al.* teach isolation and purification of human cortistatin of SEQ ID NO: 26 (page 158, Figure 1) in pharmaceutical compositions (page 158, first column) that include the pharmaceutically acceptable carrier, AcONH4 buffer (pH 8) and also comprise the pharmaceutically acceptable carrier, H_2O..."

As such, the Applicants are entitled to a conception date for pharmaceutical compositions of human cortistatin prior to March 6, 1997. To deny the Applicants the conception date of pharmaceutical compositions would not provide adequate protection for their

invention. On the contrary, it would encourage persons armed with the knowledge of the Applicants' invention to use pharmaceutical compositions of cortistatin to escape the literal language of unduly limited claims. Any competitor seeking to avoid such claims would merely have to follow the Applicants' teachings to prepare pharmaceutical compositions of human cortistatin.

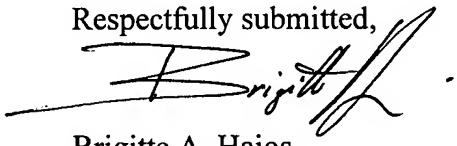
In light of the above remarks, Applicants respectfully request that the rejection of claim 21-23 under 35 U.S.C. §102(a), be withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



Brigitte A. Hajos
Reg. No. 50,971

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 415-576-0200
Fax: 415-576-0300
Attachments
BAH:bah
60323574 v1

Gene, 173 (1996) 227–232

GENE 09782

Organization of the gene encoding transcriptional repressor δEF1 and cross-species conservation of its domains

(Chicken δ-crystallin enhancer; E2 box; zinc finger; homeodomain; G+C-rich promoter)

Ryohei Sekido[a], Tsuyoshi Takagi[a], Masahiro Okanami[b], Hiroki Moribe[a], Mayumi Yamamura[a], Yujiro Higashi[a] and Hisato Kondoh[a]

[a]*Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka 565, Japan; and* [b]*Department of Botany, Faculty of Science, Kyoto University, Sakyouku, Kyoto 606, Japan. Tel. (81-71) 753-4126*

Received by A. Nakazawa: 20 October 1995; Revised/Accepted: 25 December 1995; Received at publishers: 22 February 1996

SUMMARY

δEF1 (δ-crystallin/E2-box factor 1) is a widely distributed repressor of transcription which binds at the E2-box sequence, CACCTG. It carries seven zinc fingers (Zf) in two clusters and a homeodomain in the middle as potential DNA-binding domains. We cloned the genomic gene encoding chicken δEF1 and analyzed its organization. The gene consisted of nine exons, the N-proximal Zf were encoded by exons 5 through 7, and the C-proximal Zf by exons 8 and 9. Exon 7 also coded for the large middle portion of the protein including the homeodomain. Promoter analysis and RNase-protection assay indicated that the gene is driven by a G+C-rich promoter without a TATA box, and the transcription start points (*tsp*) cluster around 20 bp from the start codon located in exon 1. cDNA and genomic sequences of the mouse *δEF1* were cloned and compared with the chicken sequence. The deduced amino acid (aa) sequence was highly conserved between the chicken and mouse δEF1, not only in DNA-binding motifs but also in other blocks (78% overall aa identity). More recently reported DNA-binding proteins, AREB6 (human), ZEB (human) and BZP (hamster), were attributed to homologues of δEF1, among which only AREB6 represented a full-length sequence. It was also indicated that rodent *δEF1* lacked exon 3.

INTRODUCTION

There is mounting evidence that an essential mechanism in cell-specific transcriptional regulation involves repression in addition to activation (Ruezinsky et al., 1991; Rahuel et al., 1992). In general, a repressor competes with an activator for binding to the same target DNA sites. For instance, many of E2-box (5'-CACCTG) or related sequences are known to be the site of competition between bHLH activators and a widespread repressor (Ruezinsky et al., 1991; Simon et al., 1993; Sekido et al., 1994).

We have studied lens-specific regulation of the gene encoding δ-crystallin (Hayashi et al., 1987; Goto et al., 1990; Funahashi et al., 1991), and found that the activity of the lens-specific enhancer core is dependent on the activator-repressor competition mechanism (Kamachi and Kondoh, 1993). We cloned a cDNA of a repressor of the δ-crystallin enhancer (Funahashi et al., 1993), and found that the repressor satisfies the conditions for a

Correspondence to: Dr. H. Kondoh, Institute for Molecular and Cellular Biology, Osaka University, Yamadaoka 1-3, Suita, Osaka 565, Japan. Tel. (81-6) 879-7963; Fax (81-6) 877-1738.

Abbreviations: aa, amino acid(s); AREB, *Atp1a1* regulatory element-binding factor; bHLH, basic helix-loop-helix motif; bp, base pair(s); BZP, β-cell Zf protein; δEF1, δ-crystallin/E2-box factor 1; *δEF1*, gene (DNA) encoding δEF1; kb, kilobase(s) or 1000 bp; Luc, luciferase; nt, nucleotide(s); PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na_3-citrate pH 7.6; SSPE, 0.15 M NaCl/0.01 M Na phosphate/0.001 M EDTA pH 7.0; *tsp*, transcription start point(s); ZEB, Zf E-box-binding protein; Zf, zinc finger(s).

PII S0378-1119(96)00185-0

widespread E2-box repressor (Sekido et al., 1994). The repressor named δEF1 (δ-crystallin/E2-box factor 1) is specific to CACCT present in the δ-crystallin enhancer core, the E2-box and in the Brachyury (T)-binding site sequence (Kispert and Herrmann, 1993), and, in fact, repressed E2-box-mediated gene activation in lymphoid and myogenic cells (Sekido et al., 1994).

The δEF1 protein molecule is fairly large (120 kDa) and carries tripartite DNA-binding motifs (Funahashi et al., 1993). From its size and from the multitude of DNA-binding motifs, we expected a complex array of functional domains to be present in the molecule. To provide fundamentals for the analysis of *δEF1* regulation in vivo and of functional domains of the encoded protein, we made the following analyses: (*i*) determination of the organization of the *δEF1* gene and the promoter, and (*ii*) comparison of chicken and mouse δEF1 aa sequences deduced from cDNA sequences. The data were also used for assessment of proteins encoded by more recently reported cDNAs from human and hamster which showed similarities to δEF1 in their aa sequences.

EXPERIMENTAL AND DISCUSSION

(a) Organization of the chicken *δEF1* gene

δEF1 genomic clones were isolated by screening chicken genomic libraries with fragments of *δEF1* cDNA used as hybridization probes. Restriction maps and cDNA hybridization data allowed us to arrange the genomic clones in a contiguous map with a gap between clones GEN48 and GEN23 (Fig. 1A). The restriction fragments detected in genomic Southern blots were also accounted for by the genomic clones isolated (data not shown), indicating that *δEF1* is a single-copy gene.

The DNA sequence of the genomic clones was determined and compared with the cDNA sequence (Funahashi et al., 1993), and the exon organization was established. The *δEF1* gene spanned over 50 kb and consisted of nine exons. The N-proximal Zf were encoded by small exons 5 through 7, and the C-proximal Zf by exons 8 and 9 (Fig. 1B). Exon 7 coded for the large middle portion of the protein between the Zf clusters and including the homeodomain. No clones overlapping with both

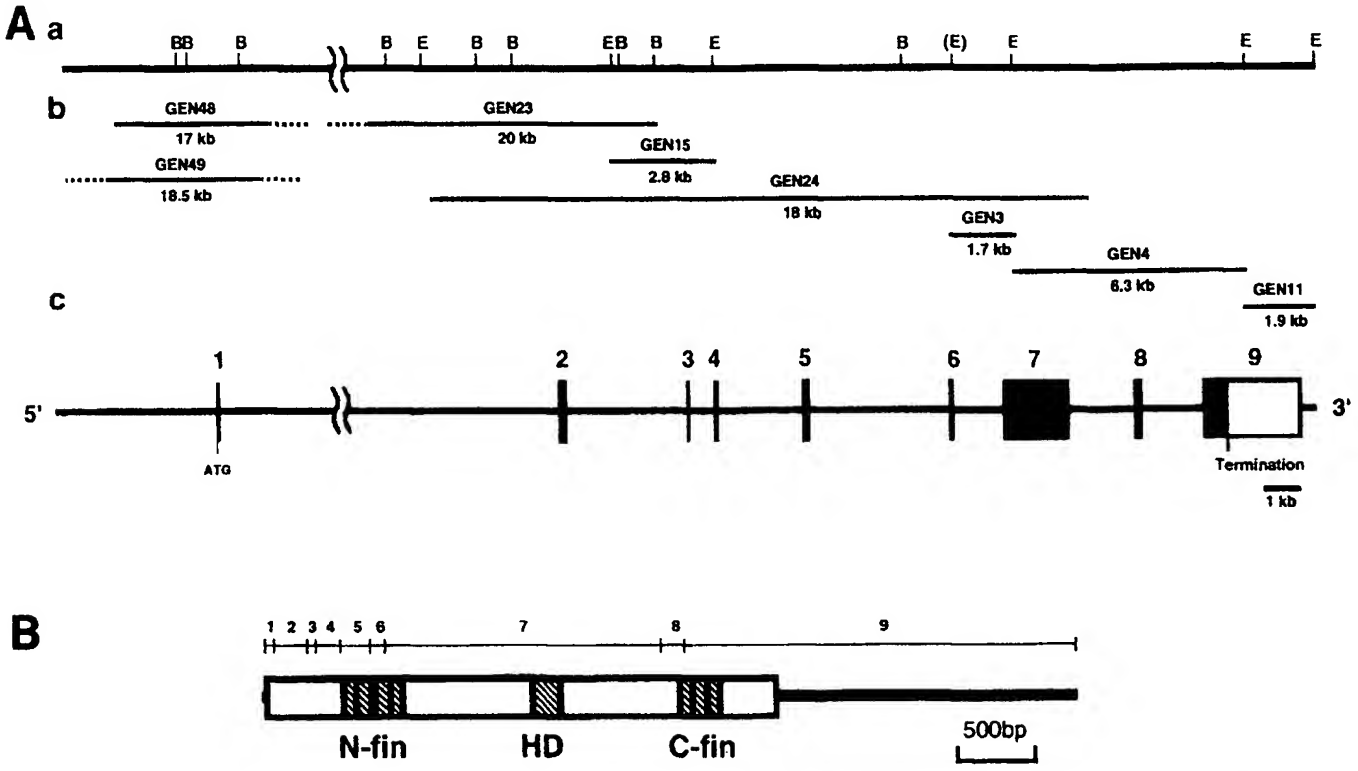


Fig. 1. Organization of the chicken *δEF1* gene. (**A**) The genomic organization of the chicken *δEF1* gene. (a) Restriction map of the gene: B, *Bam*HI; E, *Eco*RI; E in parentheses, a polymorphic *Eco*RI site. (b) The regions covered by bacteriophage clones. The lines represent bacteriophage inserts, and the clone number and the insert length in kb are indicated above and below the lines, respectively. (c) Exon organizations of the gene. Exons are shown in boxes with the numbers on top and coding regions filled. The nt sequence between the second and third *Bam*HI sites from the left and that between the fourth *Bam*HI site and the right-most *Eco*RI site in the restriction map on top are in DDBJ/GenBank/EMBL DNA databases with accession Nos. D76433 and D76434, respectively. (**B**) Allocation of the exons on the cDNA sequence as shown on top of the schematic cDNA structure. The protein-coding region of the cDNA is shown by a box in which hatches indicate potential DNA-binding domains, Zf (N-fin and C-fin) and the homeodomain (HD). **Methods:** Partial *Sau*3AI digest of chicken DNA inserted between arms of λFixII or λEMBL3, and complete *Eco*RI digest inserted between λgt10 arms were used for constructing genome libraries. The libraries were screened using *δEF1* cDNA (Funahashi et al., 1993) as probe.

GEN48 and GEN23 were obtained, indicating that exons 1 and 2 are separated by a large intron exceeding 30 kb in size.

(b) Definition of the promoter

To determine the *tsp* of the *δEF1* gene, we performed RNase protection analysis (Fig. 2A) using an antisense-RNA probe starting from a point close to the 3′ end of exon 1 and extending to the upstream region. Four major protected fragments were detected with termini mapped at 21, 16, 14 and 12 bp upstream from the start codon. Taking the T 21 upstream from the Met codon as +1, the major *tsp* were mapped at +1, +6, +8 and +10. Most of the cDNA clones thus far obtained (Funahashi et al., 1993; Y. H., unpublished observation) had their 5′ termini distributed between +3 and +11, verifying the conclusion.

The sequence upstream from the transcribed region was highly G+C-rich and devoid of a TATA box (Fig. 2B). Further upstream was found an insertion of CR1 transposon sequence (Burch et al., 1993) between −417 and −952. The promoter activity of the nt sequence upstream from +1 was assessed by transfection of cultured cells with recombinant genes. Various lengths of the upstream sequence were joined to a Luc-encoding cDNA by a bridge of exon 1 sequence from +11 to +67,

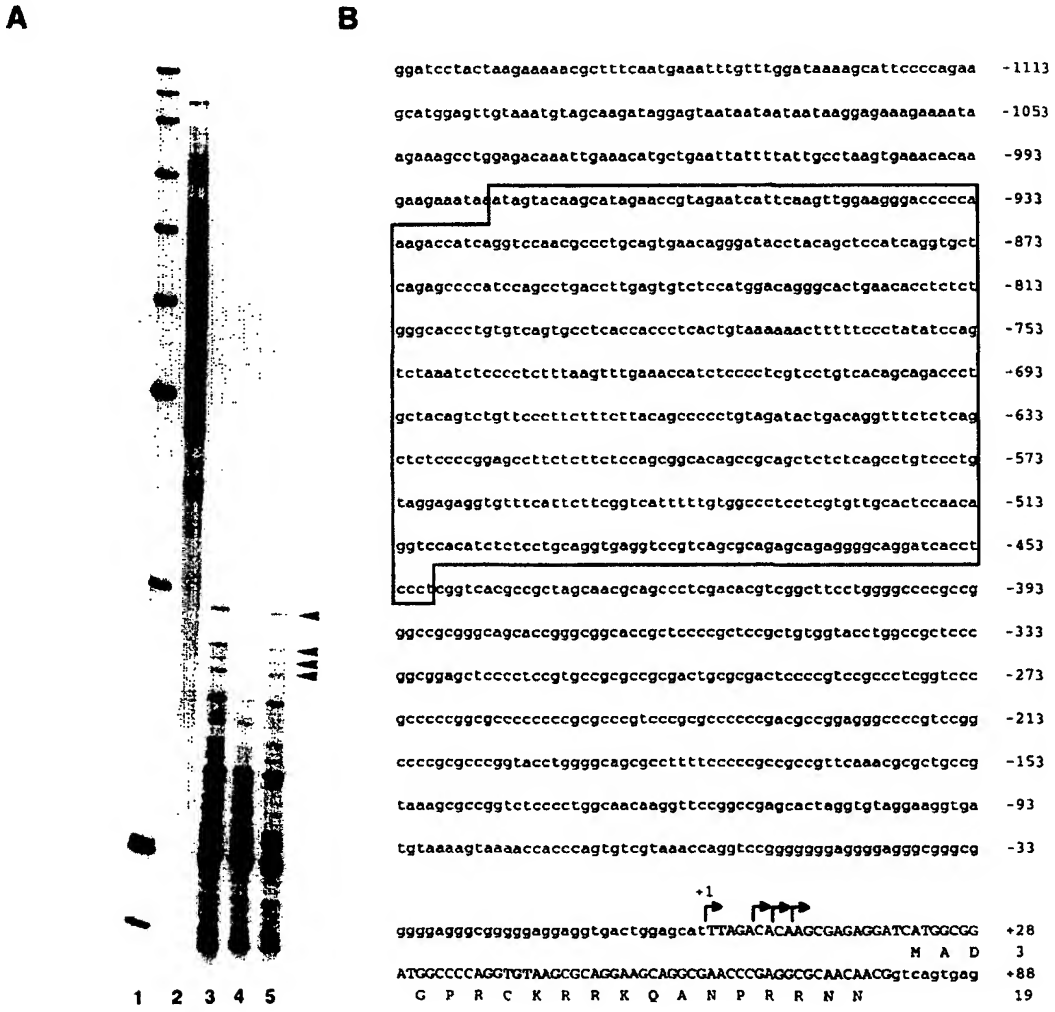


Fig. 2. The *tsp* and upstream nt sequence of the *δEF1* gene. **(A)** RNase protection analysis of the mRNA 5′-ends. Poly(A)$^+$RNA from lens (lane 3) or brain (lane 5) of 14-day chick embryo or yeast tRNA (lane 4) were hybridized with radiolabeled antisense RNA probe (lane 2), treated with RNases and the products were electrophoresed. The *Hap*II-digested pUC19 DNA (lane 1) was used as size reference. The products specific to lens or brain mRNA are indicated by arrowheads. The exact sizes of the products were determined in a separate experiment. The probe (lane 2) was once purified by polyacrylamide gel electrophoresis, but due to its high specific activity, cleavage occurred before re-electrophoresis. **(B)** The nt sequence around the *tsp* and of the upstream region. The *tsp* determined in A are indicated by arrows taking the most upstream site as +1, and the exon sequence in capital letters. The CR1 sequence is boxed. **Methods:** Poly(A)$^+$RNA was isolated from total RNA of 14.5-day chicken embryo brain using oligotex (Takara, Kyoto, Japan). Antisense 365-nt RNA probe was generated with T7 RNA polymerase (Stratagene) from a linearized plasmid carrying genomic DNA fragment from 202 nt upstream to 67 nt downstream from the start codon and labeled with [α-^{32}P]UTP (29.6 TBq per mmol) (Amersham). Protection assay was done using the RPA II Ribonuclease Protection Assay kit (Ambion, Austin, TX). RNA fragments were resolved in a 7 M urea-6% polyacrylamide gel.

and Luc was assayed in transfected primary lens cells (Fig. 3) or 10T1/2 fibroblasts (data not shown but essentially the same as Fig. 3). The promoter activity was totally dependent on this G+C-rich upstream sequence, and the nt sequence up to −73 was sufficient for full promoter activity. It has been known that such G+C-rich promoter often results in clustered, multiple *tsp* (Ishii et al., 1985; Reynolds et al., 1985; Melton et al., 1986), as observed with the *δEF1* gene. Addition of more upstream sequences which include the CR1 unit did not affect the promoter activity at all.

(c) Molecular cloning of cDNA and gene encoding mouse δEF1 and comparison of chicken and mouse δEF1

To examine the conservation of the coding sequence of the *δEF1* gene among animal species, mouse *δEF1* cDNA was cloned from a library of 12-day embryos. The sequence representing the 5′ end of the mRNA was supplemented by the RACE procedure (Frohman et al., 1988), and the entire cDNA sequence was reconstructed from the longest clones (Fig. 4A). The cDNA sequence started at position +3 and the 5′-flanking sequence of the start codon was identical between the chicken and mouse *δEF1*. The encoded aa sequences of the chicken and the mouse were aligned with a high degree of matches (78% identity). In addition, cloning and partial sequencing of the genomic clones of the mouse (data not shown) revealed that the exon-intron boundaries were the same between two animals.

It was, however, noted that the 20 aa from aa 87 to 106, corresponding exactly to exon 3 in the chicken sequence, were missing in the mouse sequence, suggesting that the mouse gene lacks this exon. In fact, the genomic sequence of the mouse contained no sequence corresponding to chicken exon 3 (data not shown).

Otherwise, aa sequence of δEF1 was conserved to a high degree between the chicken and the mouse, even outside of the putative DNA-binding domains. This suggests that this large molecule carries multiple functional domains involved in gene regulation.

(d) Relationship of other DNA-binding proteins sharing sequence similarities with δEF1

Since the full-length *δEF1* cDNA from the chicken was first reported (Funahashi et al., 1993), cDNAs coding for proteins very similar to δEF1 have been cloned from human and hamster, and given different names: AREB6 was cloned as a binding protein of the promoter of Na,K-ATPase α1 gene (Watanabe et al., 1993), ZEB (Genetta et al., 1994) as an immunoglobulin heavy-chain enhancer-binding protein, and BZP (Franklin et al., 1994) fortuitously isolated from hamster insulinoma cells as a protein binding to a synthetic probe. Aa sequences of these proteins aligned well with those of chicken and mouse δEF1 (Fig. 4A), and DNA-binding motifs were organized identically (Fig. 4B). This indicated that they are human and hamster homologues of δEF1.

There were, however, notable disparities of the struc-

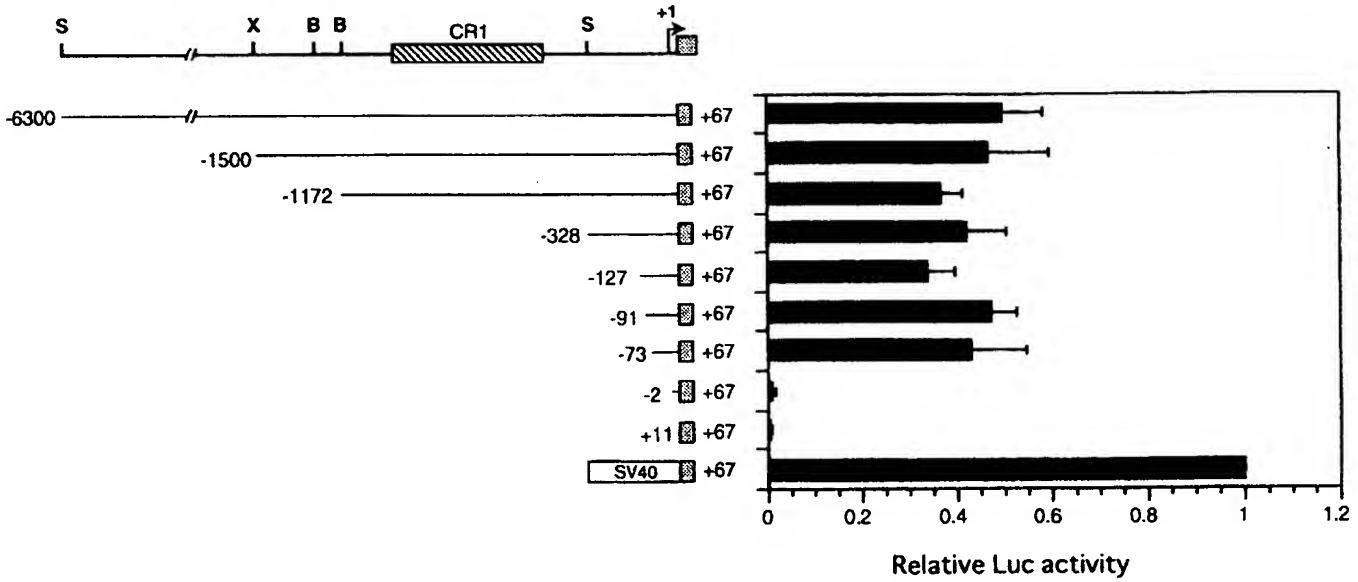


Fig. 3. Promoter activity of the 5′-flanking sequence of the *δEF1* gene. Various lengths of the 5′-flanking sequence (thin lines) or SV40 early regulatory region (open box) were joined to +11 to +67 region of the *δEF1* exon 1 sequence (shaded box) and the Luc-encoding sequence in plasmid p+11δHLuc. Numbers indicate positions in nt. The CR1 sequence is indicated by a hatched box. The plasmid DNAs carrying the constructs [1 μg of total DNA containing 0.1 pmol (0.48–1.0 μg) of the reporter plasmid and variable amounts of stuffer pUC19 plasmid] were transfected into chicken lens cells in primary culture (2×10^5), and Luc activity after 24 h was measured (Kamachi and Kondoh, 1993). Relative Luc levels based on an average of three independent transfections are shown as solid bars with the standard deviations.

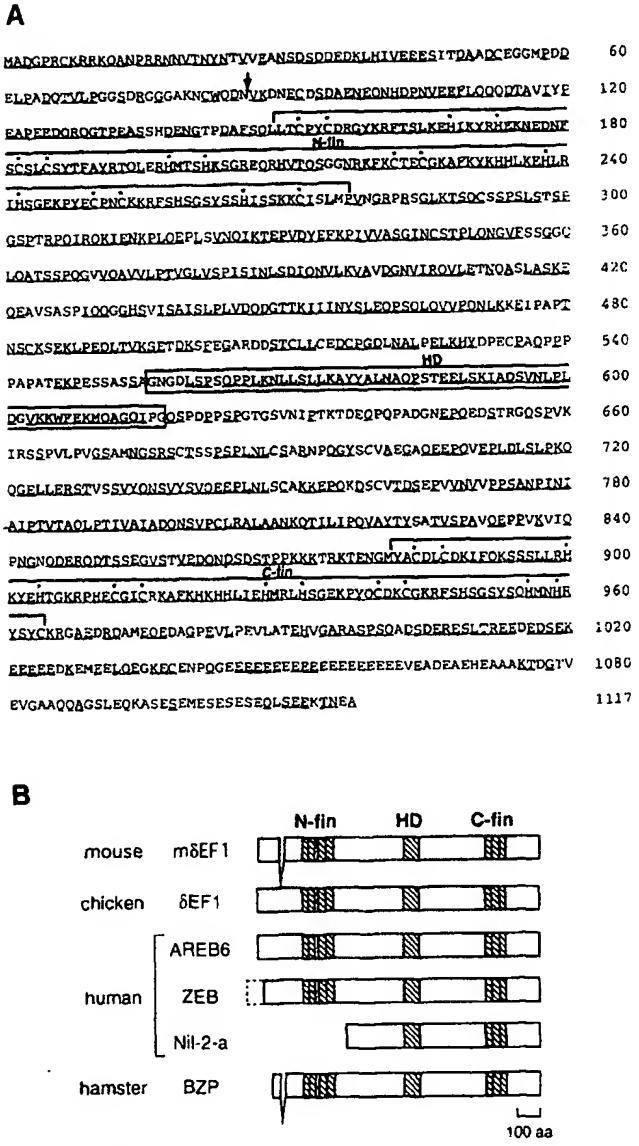


Fig. 4. Conservation of the aa sequence of δEF1 among vertebrates. **(A)** The aa sequence of mouse homologue of δEF1. The aa residues identical among vertebrates are underlined. The N-proximal and C-proximal Zf clusters (N-fin and C-fin, respectively) are indicated by overlayed bars and the Cys and His residues of the fingers by asterisks. The homeodomain is boxed. The arrow indicates where exon 3 sequence is inserted in chicken δEF1. The mouse *δEF1* cDNA and aa sequences are in DDBJ/GenBank/EMBL DNA data bases with accession No. D76432. **(B)** Comparison of the structure of cDNA-encoded proteins related to chicken and mouse δEF1. The human homologue sequence was based on AREB6 (Watanabe et al., 1993), and hamster homologue based on BZP (Franklin et al., 1994). Rodent (mouse and hamster) δEF1 lacks exon 3 sequence. **Methods:** A cDNA library of 12-day mouse embryo was constructed from oligo (dT)-primed cDNAs inserted into λgt10 arms (Tomotsune et al., 1993), and screened with chicken *δEF1* cDNAs, RS12-4 and RS12-119, as probe (Funahashi et al., 1993) under a low-stringency washing condition (3×SSC/0.1% SDS at 65°C). Oligodeoxyribonucleotide primers (5'-GTGGAATTCTGGCACTG-CCTGGTGATGTCGAAAG for reverse transcription, and 5'-AAGGAATTCCGAGGAACACTGAGATGTCTTGAGTC for PCR) hybridizable to the 5'-end region of the longest cDNA of the phage library were used for RACE procedure (Frohman et al., 1988) to clone the 5' end sequence of the mRNA.

ture of cDNA-encoded proteins close to the N terminus. Human AREB6 sequence (Watanabe et al., 1993) matches well with chicken and mouse δEF1 to the extremity of the N terminus, and probably represents the normal full size of human δEF1. By contrast, human ZEB sequence (Genetta et al., 1994) deviates from δEF1 sequence in the region close to its N terminus and has a longer N-terminal leader sequence. Among a number of *δEF1* cDNAs cloned from chicken or mouse embryos, none carried the sequence corresponding to ZEB N terminus (Funahashi et al., 1993; Y.H., unpublished observation). ZEB must represent a very rare form of δEF1, if a corresponding mRNA sequence exists in vivo. The hamster homologue BZP (Franklin et al., 1994) seems incomplete in its aa sequence at the N terminus. This hamster δEF1 is devoid of the exon 3 sequence in the same way as mouse δEF1, indicating that the rodent *δEF1* genes probably lack exon 3. Besides, human Nil-2-a lacking the N-proximal Zf cluster (Williams et al., 1991) (Fig. 4A) was an artificial translate of a partial cDNA clone and does not represent a natural form as indicated previously (Funahashi et al., 1993).

(e) Conclusions

(*1*) The chicken *δEF1* gene was determined to have nine exons, and exons 5 to 9 coded for DNA-binding motifs. The mouse gene lacked exon 3.

(*2*) The chicken *δEF1* gene was driven by a G+C-rich promoter without a TATA box.

(*3*) Comparison of aa sequence of the chicken and mouse δEF1 revealed a high overall similarity.

(*4*) Recently reported cDNAs for DNA-binding proteins, AREB6, ZEB and BZP were all assigned to human and hamster homologues of δEF1, among which only *AREB6* cDNA represented a normal full-length sequence.

ACKNOWLEDGEMENTS

We thank Dr. Yusuke Kamachi for discussions, Miss Kasumi Murai for help in DNA sequence analysis and Dr. Janet Rossant for her provision of the mouse genome library. This work was supported by research grants from the Human Frontier Science Program Organization (RG-304/33M to H.K.) and from the Ministry of Education, Science and Culture of Japan (to H.K., T.T., Y.H.). T.T. is a recipient of a JSPS Fellowship for Junior Scientists.

REFERENCES

Burch, J.B., Davis, D.L. and Haas, N.B.: Chicken repeat 1 elements contain a pol-like open reading frame and belong to the non-long terminal repeat class of retrotransposons. Proc. Natl. Acad. Sci. USA 90 (1993) 8193–8203.

Franklin, A.J., Jetton, T.L., Shelton, K.D. and Magnuson, M.A.: BZP, a novel serum-responsive zinc finger protein that inhibits gene transcription. Mol. Cell. Biol. 14 (1994) 6773–6788.

Frohman, M.A., Dush, M.K. and Martin, G.R.: Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85 (1988) 8998–9002.

Funahashi, J., Kamachi, Y., Goto, K. and Kondoh, H.: Identification of nuclear factor δEF1 and its binding site essential for lens-specific activity of the δ1-crystallin enhancer. Nucleic Acids Res. 19 (1991) 3543–3547.

Funahashi, J., Sekido, R., Murai, K., Kamachi, Y. and Kondoh, H.: δ-crystallin enhancer binding protein δEF1 is a zinc finger-homeodomain protein implicated in postgastrulation embryogenesis. Development 119 (1993) 433–446.

Genetta, T., Ruezinsky, D. and Kadesch, T.: Displacement of an E-box-binding repressor by basic helix-loop-helix proteins: implications for B-cell specificity of the immunoglobulin heavy-chain enhancer. Mol. Cell. Biol. 14 (1994) 6153–6163.

Goto, K., Okada, T.S. and Kondoh, H.: Functional cooperation of lens-specific and non-specific elements in the δ1-crystallin enhancer. Mol. Cell. Biol. 10 (1990) 958–964.

Hayashi, S., Goto, K., Okada, T.S. and Kondoh, H.: Lens-specific enhancer in the third intron regulates expression of the chicken δ1-crystallin gene. Genes Dev. 1 (1987) 818–828.

Ishii, S., Merlino, T. and Pastan, I.: Promoter region of the human Harvey ras proto-oncogene: similarity to the EGF receptor proto-oncogene promoter. Science 230 (1985) 1378–1381.

Kamachi, Y. and Kondoh, H.: Overlapping positive and negative regulatory elements determine lens-specific activity of the δ1-crystallin enhancer. Mol. Cell. Biol. 13 (1993) 5206–5215.

Kispert, A. and Herrmann, B.G.: The *Brachyury* gene encodes a novel DNA binding protein. EMBO J. 12 (1993) 3211–3220

Melton, D.W., McEwan, C., McKie, A.B. and Reid A.M.: Expression of the mouse HPRT gene: deletional analysis of the promoter region of an X-chromosome linked housekeeping gene. Cell 44 (1986) 319–328.

Rahuel, C., Vint, M.A., Lamarchandel, V., Cartron, J.P. and Romeo, P.H.: Erythroid-specific activity of the glycophorin B promoter requires GATA-1 mediated displacement of a repressor. EMBO J. 11 (1992) 4095–4102.

Reynolds, G.A., Goldstein, J.L. and Brown, M.S.: Multiple mRNAs for 3-hydroxy-3-methylglutaryl coenzyme A reductase determined by multiple transcription initiation sites and intron splicing sites in the 5'-untranslated region. J. Biol. Chem. 260 (1985) 10369–10377.

Ruezinsky, D., Beckmann, H. and Kadesch, T.: Modulation of the IgH enhancer's cell type specificity through a genetic switch. Genes Dev. 5 (1991) 29–37.

Sekido, R., Murai, K., Funahashi, J., Kamachi, Y., Fujisawa-Sehara, A., Nabeshima, Y. and Kondoh, H.: The δ-crystallin enhancer-binding protein δEF1 is a repressor of E2-box-mediated gene activation. Mol. Cell. Biol. 14 (1994) 5692–5700.

Simon, A.M. and Burden, S.J.: An E box mediates activation and repression of the acetylcholine receptor δ-subunit gene during myogenesis. Mol. Cell. Biol. 13 (1993) 5133–5140.

Tomotsune, D., Shoji, H., Wakamatsu, Y., Kondoh H. and Takahashi, N.: A mouse homologue of the *Drosophila* tumour-supressor gene *l(2)gl* controlled by Hox-C8 in vivo. Nature 365 (1993) 69–72.

Watanabe, Y., Kawakami, K., Hirayama, Y. and Nagano, K.: Transcription factors positively and negatively regulating the Na,K-ATPase α1 subunit gene. J. Biochem. 114 (1993) 849–855.

Williams, T.W., Moolten, D., Burlein, J., Romano, J., Bhaerman, R., Godillot, A., Mellon, M., Rauscher III, F.J. and Kant, J.A.: Identification of a zinc finger protein that inhibits IL-2 gene expression. Science 254 (1991) 1791–1794.